THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 13, Issue of May 5, pp. 7338-7344, 1990
Printed in U.S.A.

Expression and Characterization of a Recombinant Human Parathyroid Hormone Secreted by *Escherichia coli* Employing the Staphylococcal Protein A Promoter and Signal Sequence*

(Received for publication, January 9, 1990)

Anders Høgset‡, Ola R. Blingsmo, Olav Saether, Vigdis T. Gautvik, Erik Holmgren§, Maris Hartmanis§, Staffan Josephson§, Odd S. Gabrielsen¶, Jan O. Gordeladze, Peter Alestrøm‖, and Kaare M. Gautvik

From the Institute of Medical Biochemistry, University of Oslo, P. O. Box 1112, Blindern, 0317 Oslo 3, Norway; the §KabiGen AB, Strandbergsgatan 49, S-11287 Stockholm, Sweden, the ¶Institute of Biochemistry, University of Oslo, P. O. Box 1041 Blindern, 0317 Oslo 3, Norway, and the ‖Agricultural University of Norway, P. O. Box 36, N-1432 Ås-NLH, Norway

Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids (hPTH(1–84)). Employing the promoter and signal sequence of *Staphylococcus aureus*-protein A we have expressed hPTH in *Escherichia coli*. The expressed proteins are excreted to the growth medium, allowing for rapid and easy purification of the desired products. By amino acid sequence analysis and mass spectrometry, we have shown that the major excreted product is correctly processed human identical hPTH(1–84). The purified recombinant hPTH(1–84) stimulates adenylate cyclase activity in rat osteosarcoma cell membranes to exactly the same extent as synthetic parathyroid hormone standards, indicating that the recombinant product has full biological activity.

Human parathyroid hormone (hPTH)[1] is a peptide of 84 amino acids, secreted from the parathyroid gland. The primary translation product is a 115-amino acid preprohormone, and the prepro part is cut off during the secretion process, yielding the 84-amino acid mature hormone (1).

hPTH is a principal homeostatic regulator of blood calcium and phosphate through its actions on kidney and bone (2, 3). At chronically high secretory rates of PTH, causing sustained abnormal concentrations, bone resorption supersedes formation, resulting in the well known pathology of hyperparathyroidism. Prolonged exposure to low/moderate doses of a biologically active PTH fragment stimulates bone formation and has also been reported to be effective in the treatment of osteoporosis by inducing an anabolic response in the bone (4, 5). Thus, hPTH is a molecule of considerable interest regarding both biological and medical aspects. However, so far studies on intact hPTH have been hampered by the limited availability and the high price of the hormone. Hence a system for the efficient expression of hPTH in microorganisms would be very advantageous for the further progression of studies on hPTH and its role in bone biology and disease.

In this report we describe the use of an expression plasmid where hPTH cDNA is fused to the signal sequence of *Staphylococcus aureus*-protein A, and is under the transcriptional control of the protein A promoter (6). *Escherichia coli* transformed with this plasmid expresses hPTH and secretes several molecular species of hPTH to the periplasmic space and even to the growth medium. As judged by a series of biochemical parameters, we have achieved the expression of hPTH(1–84) as an extracellular peptide in *E. coli*, representing up to 50% of the total amount of PTH-related peptides produced, and mounting to about 1 mg/liter growth medium. The purified product had a correct amino-terminal amino acid sequence, and we could demonstrate full biological activity in an adenylate cyclase assay using rat osteosarcoma cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and other DNA-metabolizing enzymes were obtained from New England Biolabs. ^{125}I-Antirabbit-IgG was from Amersham Corp., and the NH_2-terminal-specific anti-PTH antibody was bought from CHEMICON. The production and characterization of the other antiserum has been described earlier (7). Synthetic hPTH(1–84) and [Nle^8,Nle^{18},Tyr^{34}]bPTH(1–34)amide were from Sigma.

Bacterial Strains, Plasmids, and Recombinant DNA Methods—*E. coli* strain BJ5183 (8) was obtained from Dr. F. Lacroute (Centre de Génétique Moleculaire du C. N. R. S., Gif-sur-Yvette, France). The cloning of hPTH cDNA has been described elsewhere.[2]

If not otherwise stated, recombinant DNA methods were performed according to Maniatis *et al.* (9). DNA sequencing was performed on plasmid DNA with Sequenase (United States Biochemical Corporation) according to the suppliers manual. The oligonucleotides used were synthesized with an automated machine (KabiGen AB, Sweden) as described (10).

Cell Growth and Preparation of Cellular Fractions—For testing of PTH-production, *E. coli* was grown in 2 × YT medium (16 g of Bacto Tryptone; 16 g of Bacto Yeast Extract; 10 g of NaCl/liter) containing 0.4% glucose and 0.5 g/liter of ampicillin. Cells were harvested by centrifugation at 10,000 × *g* for 10 min, and the supernatant was taken as the growth medium fraction. The periplasmic fraction was prepared by the osmotic shock method described by Nossal and Heppel (11). The soluble intracellular fraction was prepared by sonicating the cell pellet remaining after extraction of the periplasmic proteins. The cell pellet was suspended in phosphate-buffered saline, 0.05% Tween 20 and sonicated 5 × 15 s on ice in a model W-10 Sonicator (Ultrasonics). Cell debris was spun down, and the supernatant was used as the soluble intracellular fraction.

Radioimmunoassay—Radioimmunoassay of hPTH was carried out as described (7) using an antiserum reactive against epitopes between amino acids 44 and 68 in hPTH.

Polyacrylamide Gel Electrophoresis and Immunoblotting—Polyacrylamide electrophoresis in the presence of SDS (SDS-PAGE) was performed as described by Laemmli (12). Samples were solubilized in

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: human parathyroid hormone, hPTH, PTH, parathyroid hormone; bPTH, bovine PTH; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

[2] Høgset, A. (1990) *Biochem. Biophys. Res. Commun.* **166**, 50–60.

a buffer containing 0.1 M Tris-HCl, pH 7.5, 17% glycerol, 4% SDS, 0.05% bromphenol blue, and 2% β-mercaptoethanol and incubated on a boiling water bath for 5 min before being loaded on a 15% gel. Electrophoresis was run for 2–3 h at 600 V constant voltage.

For electrophoresis in the presence of acetic acid and urea, the gel was made up with a solution containing 4.5 M urea and 0.9 M HAc. Freeze-dried samples were dissolved in a sample buffer containing 0.9 M HAc, 8 M urea, 2% 2-mercaptoethanol and 0.05% pyronin Y. Electrophoresis was run in 0.9 M HAc at 180 V till the dye had migrated close to the end of the gel.

Proteins fractionated by SDS-PAGE were transferred electrophoretically to Immobilon polyvinylidene difluoride transfer membranes (Millipore) using the buffers of Towbin *et al.* (13) and a "Semi-dry Electroblotter model B" from ANCOS APS. The transfer was complete after 2 h at 0.2 A constant current. Acetic acid-urea gels were soaked three times for 15 min in transfer buffer containing 0.01% SDS, and electroblotted in the same buffer, as described above.

For staining of total proteins the filters were stained in 0.1% Coomassie-R in 50% methanol for 10 min, followed by destaining in 50% methanol, 10% HAc three times for 5 min and air drying of the filters.

For antibody probing the stained filters were soaked in methanol for a few seconds and rinsed in water for 5 min. Unspecific binding sites on the filters were blocked by incubating the filters in phosphate-buffered saline with 5% non-fat dry milk for 1 h at room temperature. Antibody incubations and washes were performed according to Towbin *et al.* (13). Cock anti-PTH antiserum that reacts with epitopes within amino acid number 44–68 (7) was used (dilution 1:8000) as a primary antibody and rabbit anti-cock-IgG (dilution 1:1000) as the secondary antibody. As a tertiary antibody we used ^{125}I-antirabbit-IgG from donkey. The NH_2-terminal specific anti-hPTH rabbit antiserum was used (dilution 1:1000) with a secondary ^{125}I-antirabbit-IgG from donkey. Autoradiography was performed overnight at −70°C with Kodak X-Omat AR5 film and an intensifying screen.

Concentration and Purification of hPTH by S-Sepharose Chromatography—Cultures (0.5 or 1 liter) were grown in 1 or 2-liter Erlenmeyer flasks at 37 °C in a shaking incubator. The cultures were grown to an OD_{600} of 2.0–2.8 and harvested by centrifugation. The pH in the supernatant (medium fraction) was adjusted to 2.95 by the addition of HCl, and the acidified medium was filtered through a Whatman GF/C filter. The filtrate (usually 5–10 liter) was applied on a column of *S*-Sepharose (Pharmacia LKB Biotechnology, Inc. column volume 300 ml) at a flow-rate of 300 ml/min. After application the column was washed by 450 ml of 0.1 M HAc, pH 6.0, and PTH was eluted with 750 ml of 0.1 M Na_2HPO_4, pH 8.5. The elution of proteins was monitored by reading the absorbance at 280 nm, total PTH in the fractions was detected by radioimmunoassay, and the molecular species were characterized by electrophoresis and immunoblotting. After elution the column was washed with 4 bed volumes of 0.1 M NaOH followed by 0.5 bed volumes of water, and regenerated by 4–5 bed volumes of 0.3 M glycine, pH 3.0.

Purification of PTH by HPLC—PTH from the peak fractions after *S*-Sepharose chromatography was further purified by reversed-phase HPLC using a Vydac RP C_{18} protein/peptide column. For small samples (up to 5 ml) a 25-cm × 4.6-mm column was used. Larger samples (5–50 ml) were chromatographed on a 30-cm × 22-mm column (Vydac, Mojave Hesperia, CA), using LDC constametric pumps model I and III, LDC gradient master, LDC spectromonitor II (LDC, Milton Roy Co, Riviera Beach, FL) and a Vitatron 2 channel recorder. The experimental conditions in the first HPLC purification were as follows. *Eluant A*, consisted of 0.1% trifluoroacetic acid in filtered and distilled H_2O. Eluant B, consisted of 70% acetonitrile in eluant A. Flow was 1.0 and 10.0 ml/min for small and large columns, respectively. Gradient was 35–55% eluant B (linear) in 48 min. Washing was with 100% B for 10 min and equilibration with 35% B. In the second HPLC purification, the small column was used under the same experimental conditions as in the first HPLC purification with the following exception: gradient was 44–49% B for 40 min.

Amino Acid Sequence Analysis—Amino acid sequencing was either done directly on fractions from the second HPLC purification, or it was performed on proteins separated by SDS-PAGE (14). In this case the proteins were blotted to a polyvinylidene difluoride filter, and the filter was Coomassie-stained as described. Interesting bands were cut out from the filter by a sterile scalpel and sequenced in a 477A protein sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA). All reagents were obtained from Applied Biosystems. Amino acid sequencing was performed by Dr. K. Sletten, Institute of Biochemistry, University of Oslo.

Mass Spectrometry—The molecular mass of purified recombinant hPTH was determined by ^{252}Cf plasma desorption mass spectrometry utilizing a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden). Recombinant hPTH (1 nmol) was dissolved in a 0.1% solution of trifluoroacetic acid in water containing 1 nmol of glutamic acid. Approximately 5 μl of this solution was applied to an aluminum sample foil coated with a thin layer of nitrocellulose and dried under a stream of nitrogen. The sample was analyzed in the mass spectrometer without rinsing the nitrocellulose layer after sample application, since rinsing completely abolished the PTH peaks in the spectrum. An acceleration voltage of 18 kV was used for collection of the spectra. Time-of-flight measurements were made with a resolution of 1 ns and converted to mass spectra using the time centroids for H^+ and Al^{2+}, respectively. Data were collected for 4 h, and the spectra were printed after background subtraction.

Adenylate Cyclase Assay—Recombinant hPTH purified on HPLC and characterized by gel electrophoresis and NH_2-terminal amino acid sequence analysis was freeze dried. It was dissolved in distilled water and diluted to yield doses capable of stimulating the adenylate cyclase of UMR 106 rat osteosarcoma cell membranes. Cell membranes were prepared and the assay carried out as previously described (15, 16). The experiments were performed in triplicate determinations which differed by less than 17%. hPTH(1–84) and [Nle^8,Nle^{18},Tyr^{34}] bPTH(1–34)-amide from Sigma were used as references.

RESULTS

Expression Plasmid Construction—An expression plasmid (pSPTH) was constructed where cDNA for hPTH(1–84) was positioned after DNA coding for the promoter and signal peptide of *S. aureus*-protein A. This plasmid should express a fusion protein consisting of the protein A signal peptide and hPTH(1–84). Thus, hPTH(1–84) could be expected to be translocated to the periplasmic space (6), and the signal peptide should be cleaved off during this process.

The construction of the expression plasmid is outlined in Fig. 1. The *Bgl*III-*Xba*I fragment containing the entire hPTH coding region was excised from the plasmid pSSHPTH10[2] and inserted between the *Bam*HI and *Xba*I sites in pUC19. This plasmid (designated pUC19PTH) was then cleaved with *Ava*I and *Xba*I and the resulting 300-base pair fragment was inserted between the *Ava*I and *Xba*I sites in pKP43 giving the plasmid pKP43PTH. To get a protein A identical signal peptide correctly positioned in front of the PTH coding sequence, pKP43PTH was cleaved by *Ava*I and *Nsi*I. A synthetic oligonucleotide (see Fig. 1) was then inserted between these sites to give the final expression plasmid pSPTH. The correct sequence of the expression plasmid was confirmed by sequencing of plasmid DNA.

Production of hPTH—Several *E. coli* strains were transformed with the expression plasmid pSPTH, and different cellular fractions were tested for hPTH production by radioimmunoassay. Of the strains tested BJ5183 gave the highest overall level of expression, and this strain was therefore chosen for a more detailed study of hPTH expression.

A time course of the PTH production in this strain transformed with pSPTH is shown in Fig. 2. While the overall production of PTH increased in parallel with the OD_{600} of the culture, the localization of hPTH-immunoreactive material changed as a function of growth time. At early growth stages most of the PTH was located in the periplasmic space, while more than 80% of the total PTH immunoreactivity had accumulated in the growth medium at the stationary phase of growth. It was also apparent that the secretion process must be very efficient in that only a very small fraction of the total hPTH-related material was found in the intracellular fraction.

Analysis of Expression Products by Polyacrylamide Electrophoresis and Immunoblotting—To analyze the expression products in more detail, we subjected proteins from the growth

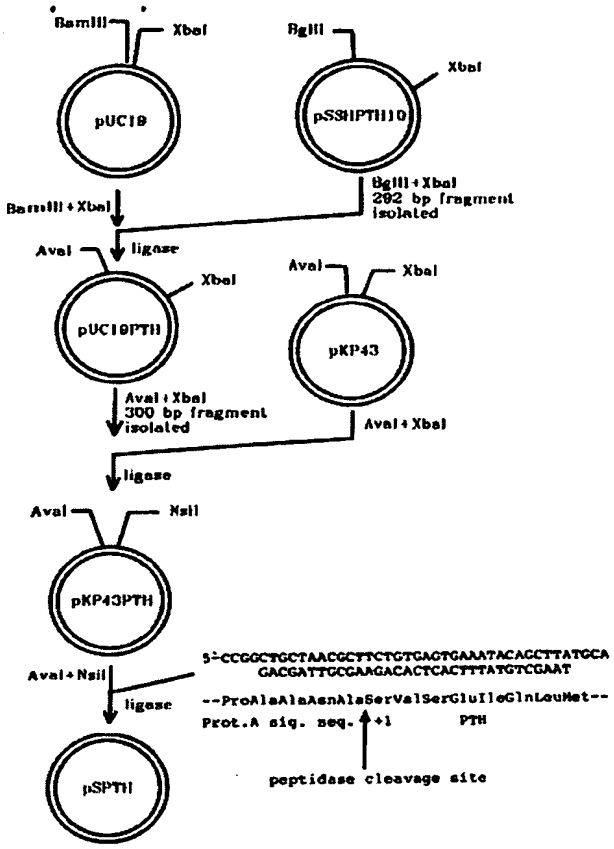

FIG. 1. Construction of the expression plasmid pSPTH. For details, see text.

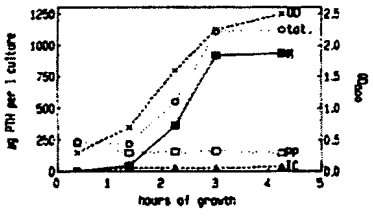

FIG. 2. hPTH production as a function of culture growth. Cells were grown as described, and the amounts of hPTH immunoreactive material in the periplasmic (*PP*), medium (*M*), and intracellular (*IC*) fractions were determined by radioimmunoassay. OD_{600} of the culture (*OD*) was determined in dilutions to about OD 0.5, and the total amount of PTH immunoreactivity produced is also shown (*tot*).

medium and the periplasmic space to SDS-PAGE and immunoblotting.

In Fig. 3 (*lane 1*) is shown an experiment where proteins from the growth medium have been subjected to immunoblotting using the middle/COOH-terminal antibody. Four major bands can be seen, one 9.5-kDa band comigrating with the PTH standard, two smaller bands with M_r values of about 6,000 and 5,500, and one larger band with an M_r of about 13,500. As discussed below some of these peptides have been purified and subjected to NH_2-terminal amino acid sequence analysis.

As can be seen from Fig. 3 (*lane 2*), four major hPTH-related peptides could be detected after immunoblotting of

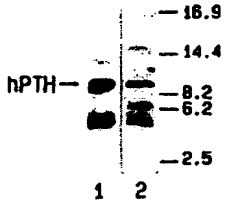

FIG. 3. Analysis of expression products by SDS-PAGE and immunoblotting: comparison of products found in the periplasmic and the medium fractions from *E. coli* transformed with pSPTH. Cells were grown, and the medium and periplasmic fractions were prepared as described. The fractions were concentrated by freeze drying, and amounts representing 100 μl of the culture were applied on the gel (*lane 1*, medium 2, periplasm). Electrophoresis and immunoblotting were performed with the middle/COOH-terminal specific antibody as described. The positions of hPTH and molecular weight standards are indicated.

the periplasmic proteins. One of these peptides had an M_r of 9500 and comigrated exactly with the hPTH standard.

The largest of the immunoreactive peptides from the periplasm has an M_r of about 14,500 and suggestively represents the signal sequence hPTH fusion protein with an uncleaved signal sequence.

The smaller immunoreactive bands probably represent proteolytic degradation products of hPTH. The major smaller bands from the periplasm have M_r values of about 6500 and 5500, and the smaller of these proteins comigrates with a protein also found in the growth medium. The 14.5-kDa band seen in the periplasmic fraction has never been observed in the growth medium fraction, indicating either that this peptide remain in the periplasm, or that it is cleaved during or after excretion to the medium. The smaller bands might correspond to cleavage of the PTH molecule at about position 25 and 35. These regions are known to be susceptible to proteolysis in other systems (17) and might be acted upon by a variety of proteases. In all experiments the predominant band is the band comigrating with the PTH standard (Fig. 3 is a representative example). However, this band generally seem to constitute a greater proportion of the total immunoreactive material in the growth medium than in the periplasm.

Purification of hPTH Species from the Growth Medium—PTH was concentrated from the growth medium by chromatography on *S*-Sepharose, and PTH in the fractions was detected as described under "Experimental Procedures" (data not shown). Fractions containing hPTH(1–84) were then subjected to reverse-phase HPLC. As shown in Fig. 4*A*, a major peak (*fractions 32* and *33*) with the same retention time as standard hPTH could be identified. Proteins from this and from the other major peak (fraction 16) were freeze-dried and subjected to SDS-PAGE and immunoblotting. As shown in Fig. 5*A* (*lanes 1* and *2*), the peak with the same retention time as hPTH mainly consists of two proteins, a main component with an M_r identical to an hPTH standard and a minor component with an M_r of about 13,500. Both of these peptides react with an anti-PTH antibody on immunoblots (Fig. 5*B*, *lanes 5* and *6*). The main component (hereafter called recombinant hPTH) was further purified by another round of reverse-phase HPLC as described. As shown in Fig. 4*B*, this procedure resolved the two components into two peaks. The major peak eluted exactly like an hPTH(1–84) standard (Fig. 4, *B* and *C*). Finally, when this peak and standard hPTH(1–84) were cochromatographed one symmetric peak appeared, indicating that the recombinant hPTH behaved exactly as the hPTH standard under these experimental conditions (Fig. 4*D*). SDS-PAGE of the peak fraction showed one band co-

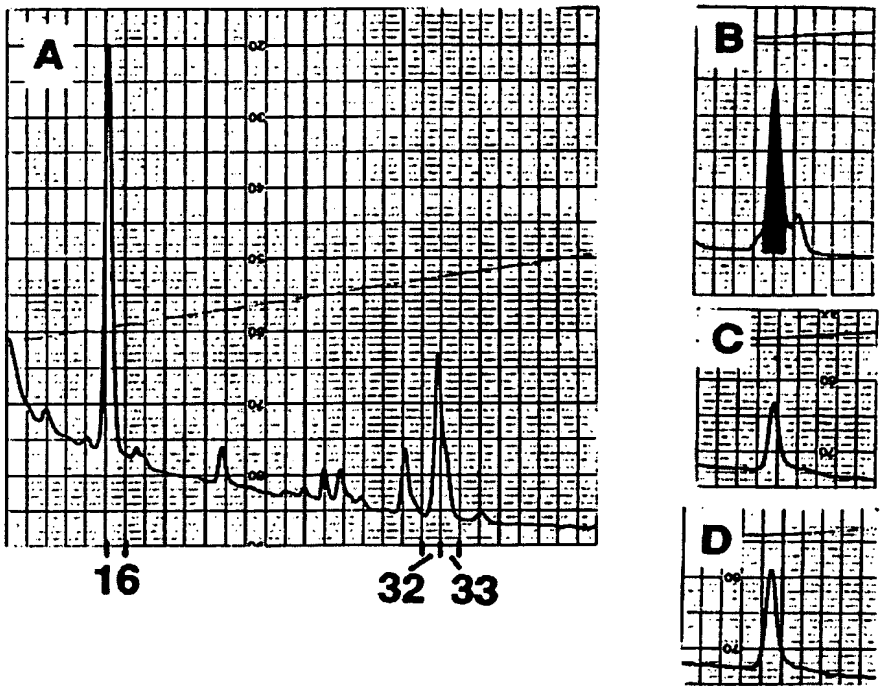

FIG. 4. **Purification of recombinant hPTH from the growth medium of *E. coli* BJ5183.** Recombinant hPTH was purified as described under "Experimental Procedures." *A*, chromatogram (A_{220}) of the first HPLC purification. Fractions used for SDS-PAGE and second HPLC purification are indicated. *B*, chromatogram of the second HPLC purification of fractions 32 and 33 from *panel A*. The peak of recombinant hPTH is indicated in *black*. *C*, second HPLC run of 1 μg of standard hPTH(1–84). *D*, cochromatography of the recombinant PTH peak from *panel B* and 1 μg of standard hPTH(1–84).

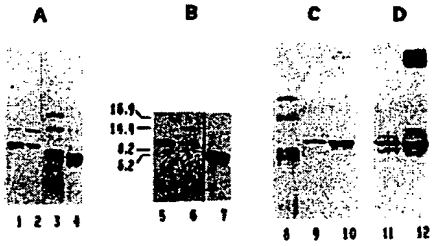

FIG. 5. **SDS-PAGE and immunoblotting of peaks from first and second HPLC purification.** Proteins from the fractions indicated in Fig. 4 were subjected to SDS-PAGE and immunoblotting as described. Molecular weight standards are indicated. *A*, Coomassie Brilliant Blue staining of the filter after SDS-PAGE and blotting of fractions from the first HPLC run. *B*, autoradiogram of an immunoprobed filter (using the middle/COOH-terminal antiserum) of the same samples electrophoresed on a parallel gel (20 times less material was loaded on this gel than on the one shown in *panel A*). *C*, Coomassie Brilliant Blue and, *D*, silver staining of the gel after SDS-PAGE of the proteins in the recombinant hPTH peak indicated in Fig. 4*B*. Loadings were as follows. *1* and *5*, fraction 32; *2* and *6*, fraction 33; *3* and *8*, molecular weight standards; *4* and *7*, fraction 16; *9* and *11*, recombinant hPTH peak, 1 μg; *10* and *12*, hPTH(1–84) standard, 3 μg.

migrating with the hPTH standard (Fig. 5, *C* and *D*), suggesting that the recombinant hPTH was essentially pure and that it behaved exactly like the hPTH standard also in this separation system.

The recombinant hPTH from the HPLC purification was then subjected to NH_2-terminal amino acid sequencing as described, and the result is shown in Table I. We were able to determine unambiguously 45 amino acids from the NH_2-terminal, and the determined sequence was identical to the known sequence of hPTH (18, 19). The sequence analysis also indicated that the recombinant hPTH was more than 90% pure after the three purification steps employed. The

TABLE I

NH_2-terminal amino acid sequences of PTH species purified from the growth medium

PTH immunoreactive peptides were purified from the growth medium and subjected to amino acid sequence analysis as described. *A*, recombinant PTH peak (Fig. 4*B*) (45 residues from the NH_2-terminal were sequenced; only the first 29 are shown in this table). *B*, 13.5-kDa band (Fig. 5*A*, *lane 2*). *C*, 6.0-kDa band (Fig. 5*A*, *lane 4*).

A:	S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q
B:	S V S E I Q L
C:	E W L R K K L Q

repetitive yield in this analysis was 94.5% for Leu in positions 7, 11, 15, 24, 28, and 41, and 94.5% for Val in positions 2, 21, 31, and 35.

To further substantiate the conclusion that the 9.5-kDa protein is intact hPTH(1–84), we performed acetic acid-urea polyacrylamide gel electrophoresis and immunoblotting on proteins from various stages of the purification. In this separation system proteins are separated according to a combination of charge and size. The results of such an experiment are shown in Fig. 6. It can be seen that the main immunoreactive protein in the fraction containing recombinant hPTH comigrates with the PTH standard also in this separation system (*lanes 2* and *5*).

To show that the purified recombinant hPTH represented the intact hormone, we performed mass spectrometry as described under "Experimental Procedures." The plasma desorption mass spectrum obtained from the recombinant hPTH adsorbed on the sample foil covered with nitrocellulose is shown in Fig. 7. A molecular mass of 9426 ± 9 daltons could be calculated from the single-charged and the double-charged molecular ions present in the spectrum. The theoretical molecular mass of hPTH calculated from the amino acid composition is 9425 daltons, thus corresponding nicely to the value determined for the recombinant hPTH by mass spectrometry.

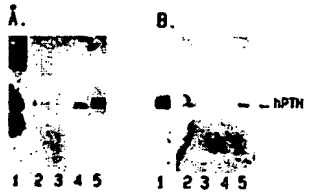

FIG. 6. **Acetic acid/urea-PAGE and immunoblotting of expression products.** Proteins were fractionated by acetic acid/urea-PAGE and blotted as described. Parallel filters were probed with the middle/COOH-terminal specific antiserum (*A*) and the NH_2-terminal specific (*B*) antibody. Loadings were as follows. *1*, proteins from the PTH-containing peak after *S*-Sepharose chromatography. 2, 50 ng of standard hPTH. 3–5, fractions 34, 33, and 32, respectively, from the first hPTH-purification (Fig. 4*A*).

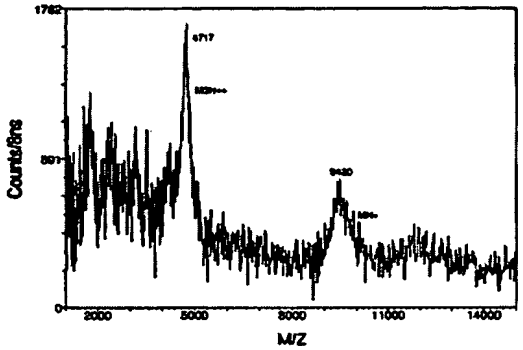

FIG. 7. **Plasma desorption mass spectrometry analysis of purified recombinant hPTH(1–84).** The spectrum shows the ion abundance as a function of mass over charge. The calculated molecular mass of native PTH is 9425 daltons.

We have also sequenced the corresponding 9.5-kDa protein isolated from the periplasm, and we found that also this protein had an NH_2-terminal amino acid sequence identical to hPTH (data not shown).

The immunoblot analysis of the HPLC-peaks also revealed two other peptides that reacted with the anti-PTH antibody, namely a 6.0-kDa peptide from fraction 16 (Fig. 4*A*, and Fig. 5, *lanes 4* and *7*) and the 13.5-kDa minor constitutent of the peak containing recombinant hPTH (Fig. 5, *lanes 2* and *6*). These peptides probably are the same as the three largest peptides detected in immunoblots of unfractionated material from freeze-dried medium shown in Fig. 3 (*lane 1*). The 6.0-kDa peptide from fraction 16 was cut out from the filter shown in Fig. 5*A* (*lane 4*) and subjected to NH_2-terminal amino acid sequence analysis. The amino acid sequence showed that this peptide was a fragment of hPTH, starting at amino acid 22 and probably extending all the way through to the COOH-terminal end of hPTH(1–84) (Table I).

The NH_2-terminal sequence of the 13.5-kDa peptide (Table I) was determined by cutting out the Coomassie-stained band after SDS-PAGE and blotting of proteins from the minor peak from the second HPLC run (Fig. 4*B*).

As shown in Table I, this peptide has an NH_2-terminal amino acid sequence that is identical to that of hPTH, despite being about 4 kDa larger than hPTH(1–84). At present the molecular identity of this 13.5-kDa peptide is unclear. It is interesting to note, however, that an hPTH-immunoreactive peptide with the same M_r is observed also when hPTH is expressed as an intracellular peptide in *E. coli*.[2] Thus, the production of the 13.5-kDa peptide does not seem to be a consequence of the expression of hPTH as a secreted peptide

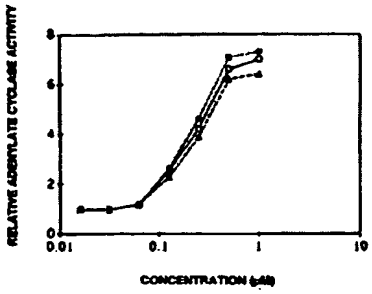

FIG. 8. **Adenylate cyclase assay of purified recombinant hPTH.** Protein from the recombinant hPTH peak indicated in Fig. 4*B* was tested in the adenylate cyclase assay as described. The basal adenylate cyclase activity was chosen as 1.0 on the ordinate. The amount of PTH was determined by radioimmunoassay of the same solution as used in the adenylate cyclase assay. (▲---▲) hPTH(1–84) standard, (○——○) recombinant hPTH, (■·····■) [Nle^8,Nle^{18},Tyr^{34}]bPTH(1–34)amide standard.

but might possibly be due to a readthrough of the PTH stop codons or to some kind of covalent modification of hPTH introduced inside the *E. coli* cell.

Biological Activity—The purified recombinant hPTH was tested for biological activity in the adenylate cyclase assay as described. It can be seen that the recombinant hPTH stimulated adenylate cyclase to the same extent as the hPTH(1–84) and [Nle^8,Nle^{18},Tyr^{34}]bPTH(1–34)amide standards, indicating that the recombinant hPTH has a specific biological activity that in this assay is indistinguishable from that of the standards (Fig. 8).

DISCUSSION

To our knowledge this work represents the first report on the successful expression and secretion of intact hPTH from *E. coli*. Other investigators have expressed hPTH as an intracellular peptides in *E. coli* (20, 21, 36) or have tried to achieve secretion of hPTH from *E. coli* using hPTH's own signal sequence (22). In the last report three forms of PTH were produced. One of these, hpreproPTH, became associated with the outside of the inner membrane, while the two other forms, hPTH(3–84) and hPTH(8–84), were intracellular peptides. The authors concluded that the hPTH signal sequence is able to confer translocation of hPTH through the inner membrane but that this signal peptide is not cleaved off by the signal peptidase, causing hpreproPTH to remain in the inner membrane.

In contrast, the protein A signal sequence used in the present work seems to be very effective in translocating hPTH through the inner membrane, indicated by the observation that only a few percent of the total hPTH immunoreactivity was located in the intracellular fraction. It also seems that the signal sequence is efficiently cleaved off during secretion. Although molecules probably corresponding to the uncleaved fusion protein could be detected in the periplasmic fraction by immunoblotting, these molecules constituted only a small amount of the total immunoreactive peptides.

A very unexpected finding was that a large amount of the immunoreactive material was excreted to the growth medium. The reason for this is at the moment unclear. It is not, however, due to cell lysis or a general leakage phenomenon because the ratio of hPTH to total protein is much higher (about 15 times, data not shown) in the medium than in the periplasmic fraction. Also the fact that the hPTH species in the medium are partly different from those seen in the periplasmic fraction (see below) argues against a generally unspe-

cific leakage of material from the cell.

Normally *E. coli* excretes very few proteins to the growth medium. The mechanism for excretion is largely unknown, but, at least in some cases, it does not seem to involve consensus signal sequences (23). In a few cases heterologous proteins have, however, been reported to be excreted after expression as fusion products with bacterial signal sequences (24–28). Excretion has also been achieved by making fusions of heterologous proteins and the signal sequence and a modified part of *S. aureus*-protein A (29–31). In this case it seems like the modified protein A part in some way makes the outer membrane leaky, so that periplasmic proteins can escape to the growth medium. The protein A signal sequence in itself does not generally seem to be able to translocate proteins to the growth medium since, for example, a fusion of this signal sequence to alkaline phosphatase expresses alkaline phosphatase as a periplasmic protein only (29). This indicates that hPTH or the fusion protein might have a structure that enables it to pass relatively easily through the outer membrane of *E. coli*, either passively or via some kind of transport system. The processes responsible for the release of intact hPTH to the medium seem to be discriminate, since some of the hPTH species in the growth medium are different from those in the periplasmic fraction and vice versa. For example, is the putative complete 14.5-kDa fusion protein never found in the medium, indicating that this form either is unable to pass through the outer membrane, or that it is in some way processed during or after translocation?

Likewise is the 13.5-kDa hPTH-form not found in the periplasmic space, suggesting that this form is either rapidly degraded in this compartment, or that it is efficiently exported to the growth medium?

The smaller PTH species observed are probably produced during or after secretion, presumably by proteases different from the signal peptidase. Such degradation of secreted proteins in *E. coli* has been described before (32), and the observation that the smaller PTH species could not be observed when PTH is expressed as an intracellular product,[2] supports the notion that the degradation in some way is associated with the secretory process.

It is at the moment unclear whether the PTH excreted passes through the periplasmic space or if it is excreted directly through the inner and the outer membrane at the same time. The fact that at least a fraction of the excreted PTH is correctly processed (presumably by signal peptidase I, see for example Ref. 33), may however, indicate that at least some of the PTH molecules go through the usual pathway for secretion of proteins to the periplasm before being excreted to the growth medium.

Our approach for producing recombinant hPTH in *E. coli* seems to have several advantages over those previously reported where hPTH has been expressed as an intracellular protein (20, 21, 36). In some of these studies, the production of hPTH was low, partly because intracellular hPTH is rapidly degraded (20, 21). In the present work the hPTH production was 10–50 times higher than what was reported in these studies. The reason for this may be that heterologous proteins tend to be more stable when released to the periplasm which may contain less proteolytic activity than the intracellular compartment (34). Even more favorable conditions may exist for proteins released to the growth medium explaining why intact hPTH accumulates in the medium during growth. Such an accumulation of hPTH could not be seen in the intracellular or periplasmic fractions, indicating a more rapid proteolytic turnover of hPTH in these compartments or escape by secretion.

Wingender *et al.* (36) recently reported on high yields when hPTH was expressed as an intracellular fusion protein. These authors, however, were unable to produce human identical hPTH(1–84), their main product being Pro-hPTH.

Expression of hPTH as a secreted protein also has the advantage of avoiding the problem with the NH_2-terminal formyl-methionine residue necessary for initiation of translation. Although this formyl-methionine residue can be removed from intracellular proteins by a deformylase and a methionine-amino-peptidase, this often is an inefficient process for heterologous proteins especially when these are highly expressed (35). In the case of hPTH, it has been shown that the removal of this residue is incomplete, even at low expression levels (21). When hPTH is expressed as a secreted protein the NH_2-terminal part of the fusion protein is cleaved off by the signal peptidase, and as long as this cleavage occurs at the right position, the NH_2-terminal of the heterologous protein should be correct. A correct NH_2-terminal is utterly important for hPTH because the biological activity of this hormone is critically dependent on the amino-terminal sequence. For example will the addition or deletion of only 1 amino acid residue usually destroy most of the biological activity (2, 3), although it very recently has been shown that Pro-hPTH has full biological activity (36)? In our expression system the hPTH fusion protein is at least partly correctly processed to hPTH(1–84) during secretion to the growth medium. In addition to hPTH(1–84), however, several hPTH fragments are produced, indicating a certain level of degradation during the secretion process.

Another obvious advantage of our expression system is the ease by which the recombinant hPTH can be purified. As can be seen from Figs. 4 and 5, the recombinant hPTH can be judged to be more than 80% pure after just two purification steps, and the sequence analysis indicates that it is more than 90% pure after the third purification step. This is in contrast to the lengthy purification procedure employed by Rabbani *et al.* (21) to purify recombinant hPTH expressed as an intracellular peptide in *E. coli*.

In conclusion we have succeeded in expressing and purifying recombinant hPTH in *E. coli*. Since the purified 9.5-kDa protein comigrates with an hPTH(1–84) standard in three different separation systems, has the right NH_2-terminal amino acid sequence, and has a correct molecular mass as determined by mass spectrometry, it seems reasonable to conclude that this protein represents intact hPTH(1–84) produced and excreted in *E. coli*. Furthermore, the purified product has full biological activity as determined in the adenylate cyclase assay. In comparison to other published systems for the expression of hPTH in microorganisms, our system gives higher production and easier purification, since the product is excreted to the growth medium. By using this expression system combined with high density fermentation methods, it should now be possible to produce pure hPTH(1–84) in such quantities that physiological and clinical studies using the intact hormone could be performed on a larger scale.

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